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Production of Monoclonal Antibodies against *Rickettsia massiliae* and Their Use in Antigenic and Epidemiological Studies

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Rickettsiae are gram-negative, obligate intracellular bacteria which have historically been divided into three groups: the typhus group, the scrub typhus group, and the spotted fever group (SFG). Recently, several new SFG rickettsiae have been characterized, and most of these species are associated with ticks and have, as yet, no known pathogenicity toward humans. *Rickettsia massiliae*, which is widely distributed in Europe and Africa, is one such rickettsia. In order to investigate the antigenic relationships between *R. massiliae* and other rickettsial species and to develop a more convenient methodology for identifying *R. massiliae*, we produced monoclonal antibodies against the type strain (Mtu1^T) of *R. massiliae* by fusing immunized splenocytes with SP2/0-Ag14 myeloma cells. A panel of 16 representatives were selected from the 163 positive hybridomas identified on initial screening, and their secreted monoclonal antibodies were further characterized. The reactivities of these 16 monoclonal antibodies with a large panel of rickettsial species were assessed by the microimmunofluorescence assay. All species of the SFG rickettsiae reacted with the monoclonal antibodies directed against epitopes on lipopolysaccharide, which is the common antigen among the SFG rickettsiae. Some closely related species of the SFG, such as Bar29, "*R. aeschlimanni*," and *R. rhipicephali*, showed strong cross-reactivities with the monoclonal antibodies directed against epitopes on the two major high-molecular-mass heat-labile proteins (106 and 120 kDa). In addition, species-specific monoclonal antibodies demonstrated that *R. massiliae* is antigenically different from other rickettsial species. Moreover, these species-specific monoclonal antibodies were successfully used for identifying *R. massiliae* in the ticks collected from southern France, and are therefore potentially useful tools in the identification and investigation of *R. massiliae* in ticks in large-scale field work.

Rickettsiae are gram-negative, obligate intracellular bacteria which are associated with arthropods. On the basis of the differences in etiology, serology, epidemiology, and intracellular growth characteristics, the genus *Rickettsia* has traditionally been divided into three different groups, namely, the typhus group, the scrub typhus group, and the spotted fever group (SFG). The typhus group rickettsia comprises three species, *R. prowazekii*, *R. typhi*, and *R. canada*, whereas the scrub typhus group rickettsia includes only one species, *Orientia tsutsugamushi* (49). The SFG is the largest rickettsial group, containing numerous antigenically related species (9).

Some SFG rickettsiae have been implicated in human disease and are therefore defined as the pathogenic species. These rickettsiae include *R. rickettsii* (the causative agent of Rocky Mountain spotted fever), *R. conorii* (Mediterranean spotted fever), *R. sibirica* (North Asian tick typhus), *R. australis* (Queensland tick typhus), *R. akari* (rickettsialpox), *R. japonica* (Japanese spotted fever), *R. africae* (African tick-bite fever), Israeli tick typhus rickettsia (Israeli spotted fever), Astrakhan fever rickettsia (Astrakhan spotted fever), and *R. honei* (Flinders Island spotted fever) (7, 31, 50, 56). Other species of SFG have been found only in ticks and have no known pathogenicity toward humans. The rickettsiae of unknown pathogenicity include "*R. aeschlimanni*," Bar29, *R. helvetica*, *R. massiliae*, *R. montana*, *R. parkeri*, *R. rhipicephali*, "*R. slovaca*," and Thai tick typhus rickettsia (9, 10).

In recent years, with the introduction of improved techniques for their isolation and identification (22, 36, 44, 46–48), numerous novel SFG rickettsiae have been identified, but their pathogenicities toward humans remain unclear (5, 11, 12). *R. massiliae* is such a species which was originally isolated from *Rhipicephalus turanicus* ticks collected from regions near Marseille, France (11, 13). Subsequently, an isolate of this species had also been found in *Rhipicephalus sanguineus* ticks in Greece (5). *R. turanicus* ticks collected in Portugal (6) and *Rhipicephalus mushamae*, *Rhipicephalus lunulatus*, and *Rhipicephalus sulcatus* ticks collected in the Central African Republic (51) have also now been shown to be infected with *R. massiliae*, indicating that the species is widely distributed. Because shell vial centrifugation isolation methods (36) and molecular biology-based identification schemes (22, 44, 46, 47) for *R. massiliae* are laborious in large-scale field work, we aimed to develop a more convenient, monoclonal antibody-based methodology to facilitate the detection of *R. massiliae* in ticks. Additionally, phenotypic and genotypic characterization of *R. massiliae* has suggested that this species is closely related to the other rickettsial species, such as Bar29, "*R. aeschalimanni*," and *R. rhipicephali* (12, 47, 48). Therefore, we also aimed to investigate the antigenic relationships between *R. massiliae* and other rickettsial species, using monoclonal antibodies produced against Mtu1, the type strain of *R. massiliae* (13).

MATERIALS AND METHODS

Rickettsiae. The sources of all strains used in this study are presented in Table 1.

The following strains were obtained from the American Type Culture Collection (ATCC; Rockville, Md.): *R. akari*, *R. conorii*, *R. rickettsii*, *R. prowazekii*, *R. typhi*, *O. tsutsugamushi*, and *Coxiella burnetii*. *R. africae*, *R. massiliae* Mtu1^T and

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TABLE 1. Rickettsial strains studied

Rickettsia[a]	Group[b]	Strain	Source	Geographic origin	Pathogenicity	Standard strain no.	Reference
Rickettsia massiliae	SFG	Mtu1^T	*Rhipicephalus turanicus*	France		ATCC VR 1376	13
Rickettsia massiliae	SFG	GS	*Rhipicephalus sanguineus*	Greece			5
**"Rickettsia aeschlimanni"*	SFG	MC 16	*Hyalomma marginatum*	Morocco			10
Rickettsia africae	SFG	Z9-Hu	Human	Zimbabwe	African tick-bite fever		32
Rickettsia akari	SFG	MK (Kaplan)	Human	New York State	Rickettsialpox	ATCC VR 148	29
*Astrakhan fever rickettsia	SFG	Human	Human	Russia	Astrakhan fever		23
Rickettsia australis	SFG	Philips	Human	Australia	Queensland tick typhus		4
*Bar29	SFG	Bar 29	*Rhipicephalus sanguineus*	Spain			12
Rickettsia conorii	SFG	Moroccan	Unknown	Morocco	Mediterranean spotted fever	ATCC VR 141	16
Rickettsia helvetica	SFG	C9P9	*Ixodes ricinus*	Switzerland		ATCC VR 1375	14
Rickettsia honei	SFG	RB	Human	Australia	Flinders island spotted fever		8
*Israeli tick typhus rickettsia	SFG	ISTT CDC 1	Human	Israel	Israeli spotted fever		27
Rickettsia japonica	SFG	YH	Human	Japan	Oriental spotted fever	ATCC VR 1363	53
Rickettsia montana	SFG	M/5-6	*Microtus* spp.	Montana			17
Rickettsia parkeri	SFG	Maculatum 20	*Amblyomma maculatum*	Mississippi			15
Rickettsia rhipicephali	SFG	3-7-6	Rhipicephalus sanguineus	Mississippi			20
Rickettsia rickettsii	SFG	Sheila Smith	Human	Montana	Rocky Mountain spotted fever	ATCC VR 149	15
Rickettsia sibirica	SFG	246	*Dermacentor nuttalli*	Former USSR	North Asian tick typhus	ATCC VR 151	16
**"Rickettsia slovaca"*	SFG	13-B	*Dermacentor marginatus*	Slovakia			54
Thai tick typhus rickettsia	SFG	TT-118	*Ixodes* and *Rhipicephalus* spp.	Thailand		ATCC VR 599	45
Rickettsia canada	TG	2678	*Haemophysalis leporispalustris*	Canada		ATCC VR 610	39
Rickettsia prowazekii	TG	Breinl	Human	Poland	Epidemic typhus	ATCC VR 142	37
Rickettsia typhi	TG	Wilmington	Human	North Carolina	Murine typhus	ATCC VR 144	37
Orientia tsutsugamushi	STG	Gilliam	Human	Japan	Scrub typhus	ATCC VR 312	18
Rickettsia bellii		369L42-1	*Dermacentor andersoni*	Ohio			42
Coxiella burnetii		Nine Mile Q	*Dermacentor andersoni*	Montana	Q fever	ATCC VR 616	21

[a] The unrecognized and unnamed species are marked with asterisks.
[b] SFG, spotted fever group; TG, typhus group; STG, scrub typhus group.

TABLE 2. Properties of monoclonal antibodies to *R. massiliae* Mtu1[T]

Monoclonal antibody	Class and subclass	Specificity[a]	Titer[b]	Reactivity in micro-IF assay with the antigen of the following species[c]:																										
				Mas	GS	Bar	Aes	Rhi	Jpn	Itt	Afr	AF	Aka	Aus	Con	Hel	Hon	Mon	Par	Ric	Sib	Slo	Ttt	Pro	Typ	Can	Bel	Tsu	Cox
MA1-B12	IgG2a	120	256	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−		−	−	−		−	−	−	−	−
MA6-F3	IgG2a	120	655,360	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA8-G2	IgG3	120	1,024	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA4-C11	IgG2b	120	327,680	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA12-G4	IgG3	120	1,024	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA2-E2	IgG1	106	2,048	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA10-D4	IgG2a	120	1,024	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA2-A1	IgG2a	106	1,024	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA12-G12	IgG2b	120	163,840	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA8-F6	IgG2a	120	163,840	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA10-E5	IgG1	120	1,024	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA10-A12	IgG2b	120	2,048	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA7-A11	IgG2a	120	327,680	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MA7-B12	IgM	LPS-like	40,960	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
MA1-B8	IgM	LPS-like	256	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
MA1-D2	NT[d]	NI	64	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Total[e]	16	16	16	16	16	9	8	6	6	5	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1	1	0	0

[a] The numbers indicate molecular sizes (in kilodaltons) of the protein on which the epitope is recognized; LPS-like, the LPS-like antigen; NI, not identified by Western immunoblotting.

[b] The homologous titers to *R. massiliae* Mtu1[T] of hybridoma culture supernatants and ascitic fluids (underlined) by the micro-IF assay.

[c] +, reactivity by the micro-IF assay; −, no reactivity by the micro-IF assay. The rickettsial species tested were *R. massiliae* Mtu1[T] (Mas), *R. massiliae* GS (GS), Bar29 (Bar), "*R. aeschlimanni*" (Aes), *R. rhipicephali* (Rhi), *R. japonica* (Jpn), Israeli tick typhus rickettsia (Itt), *R. africae* (Afr), Astrakhan fever rickettsia (AF), *R. akari* (Aka), *R. australis* (Aus), *R. conorii* (Con), *R. helvetica* (Hel), *R. honei* (Hon), *R. montana* (Mon), *R. parkeri* (Par), *R. rickettsii* (Ric), *R. sibirica* (Sib), "*R. slovaca*" (Slo), Thai tick typhus rickettsia (Ttt), *R. prowazeki* (Pro), *R. typhi* (Typ), *R. canada* (Can), *R. bellii* (Bel), *R. tsutsugamushi* (Tsu), and *C. burnetii* (Cox).

[d] NT, not typeable.

[e] The total number of monoclonal antibodies showing cross-reactivity with screening antigen.

GS, "*R. aeschlimanni*," Bar29 strain, and Astrakhan fever rickettsia were isolated in our laboratory. *R. australis*, Israeli tick typhus rickettsia, *R. rhipicephali*, "*R. slovaca*," and Thai tick typhus rickettsia were kindly provided by G. A. Dasch (Naval Medical Research Institute, Bethesda, Md.). *R. bellii*, *R. honei*, *R. japonica*, *R. montana*, and *R. parkeri* were kindly provided by D. H. Walker (University of Texas, Galveston). *R. helvetica* was kindly supplied by W. Burgdorfer (Rocky Mountain Laboratory, Hamilton, Mont.). *R. sibirica* and *R. canada* were obtained from the Gamaleya Research Institute (Moscow, Russia).

Preparation of antigen. Typhus group rickettsiae, SFG rickettsiae, and *C. burnetii* were cultivated in L929 cell monolayers (ATCC CCL 1 NCTC clone 929) as described previously (60). The infection of cells was monitored by Gimenez staining (26). *O. tsutsugamushi* was propagated in L929 cell monolayers at 35°C supplemented with Dulbecco modified Eagle's minimal essential medium (Eurobio, Les Ulis, France) containing 1% (wt/vol) glucose (Gibco BRL, Life Technologies Ltd., Paisley, Scotland), 10% fetal bovine serum (Gibco BRL), and 2 mM L-glutamine (Gibco BRL). Giemsa staining was performed to monitor the infection of cells.

When the cells were heavily infected, they were harvested with glass beads and stored at −80°C. These unpurified strains were directly used as antigens for the microimmunofluorescence (micro-IF) assay (60). *R. massiliae* and other rickettsiae were prepared for immunization or sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by further treating harvested cells by sonication, followed by purification by passage through a 25% (wt/vol) sucrose and a 28 to 45% (vol/vol) Renografin density gradient as described previously (59, 60).

Production of monoclonal antibodies. Purified *R. massiliae* was used as the immunogen, and 6-week-old female BALB/c mice were used to provide immunized splenocytes for hybridoma formation (28, 60). After three intraperitoneal injections with $\sim 2 \times 10^4$ organisms at 1-week intervals, mice were boosted with $\sim 4 \times 10^3$ organisms by tail vein injection. Three days later, the mice were sacrificed and their splenocytes were fused with SP2/0-Ag14 myeloma cells by using 50% (wt/vol) polyethylene glycol (molecular weight, 1,300 to 1,600; Sigma Chemical Co., St. Louis, Mo.) (28). The fused hybridoma cells were distributed into 96-well plates (Nunc, Roskilde, Denmark) and were grown with 1× hypoxanthine, aminopterin, and thymidine selection medium (Sigma). Ten days after fusion, supernatants were recovered from wells containing cloned cells. The presence of antibodies was determined by a micro-IF assay incorporating the set of antigens of *R. massiliae*, *R. africae*, *R. australis*, *R. conorii*, *R. rickettsii*, and *R. typhi* (see below). Positive hybridomas exhibiting different reactivities with screening antigens were selected and subcloned by limiting dilution (28). The immunoglobulin class and subclass of each monoclonal antibody derived from hybridomas were determined by using an immunotype mouse monoclonal antibody isotyping kit (stock no. ISO-1; Sigma). Ascitic fluid was produced by inoculating hybridomas into BALB/c mice by standard methods (28).

Micro-IF assay. Antigens were applied into wells of 24-well microscope slides with a pen nib and were then fixed in acetone for 20 min at room temperature. The micro-IF assay was performed as described previously (60). Briefly, antigen slides with hybridoma culture supernatants diluted 1:4 in phosphate-buffered saline (PBS; pH 7.3; Gibco BRL) were incubated in a humidified chamber at 37°C for 30 min, followed by three washes in PBS for 3 min each time. The dried slides were then incubated with dichlorotriazinyl amino fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) and IgM (heavy and light chains; AffiniPure; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted 1:200 in PBS in a humidified chamber at 37°C for 30 min, followed by three washes in PBS as described above. The stained slides were air dried, mounted with Fluoprep solution (BioMérieux, Marcy l'Etoile, France), and examined by epifluorescence microscopy (Axioskop 20; Carl Zeiss, Göttingen, Germany) at ×400 magnification. Pooled polyclonal antisera from immunized mice used for hybridoma formation and pooled sera from uninfected mice were used as positive and negative controls, respectively.

SDS-PAGE and Western immunoblotting. Purified organisms were suspended in an equal volume of Laemmli buffer (0.625 M Tris [pH 8.0], 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, and 0.002% [wt/vol] bromophenol blue) and then subjected to SDS-PAGE (33) in a 3.0% stacking gel with 0.5% SDS and a 7.5% resolving gel. Prestained SDS-PAGE standards (low- and high-range; Bio-Rad, Richmond, Calif.) were included in each run for estimating protein band size.

Western immunoblotting was carried out after the resolved polypeptides had been transferred onto a nitrocellulose membrane (0.45-μm pore size; Trans-Blot Transfer Medium; Bio-Rad) (60). Briefly, nonspecific binding sites were blocked by incubating membranes overnight with 5% (wt/vol) non-fat powdered milk in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5], 250 mM NaCl, 0.01% [wt/vol] Merthiolate) at room temperature, and then the membranes were washed three times with TBS for 10 min each time. Membrane strips were then incubated with different hybridoma supernatants diluted 1:8 in PBS or ascitic fluids diluted 1:1,000 in PBS for 2 h at room temperature and washed as described above. The strips were then incubated at room temperature for 1 h with peroxidase-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (heavy and light chains; AffiniPure; Jackson ImmunoResearch) diluted 1:200 in TBS containing 3% (wt/vol) non-fat powdered milk. The strips were washed again, and the bound peroxidase was detected by using the substrate mixture (60). Controls were used as described above for the micro-IF assay.

Detection of rickettsiae in tick samples. Twenty liquid nitrogen-stored *R. sanguineus* ticks, which had been collected from areas in southern France, including Marignane Massa, Vinon Sur Verdon, Marseille, and S[T] Roch, were tested for the presence of *R. massiliae*. The salivary glands were dissected from each tick, and smears were prepared as described previously (60) and then fixed

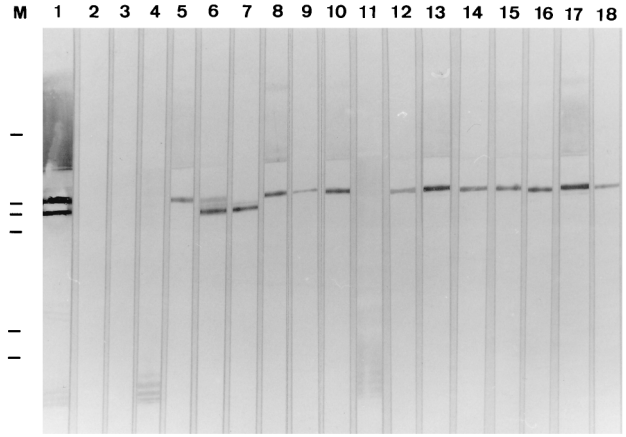

FIG. 1. Western immunoblotting of native proteins of *R. massiliae* Mtu1^T with mouse polyclonal antisera and monoclonal antibodies. Lane 1, mouse polyclonal antisera; lane 2, noninfected mouse sera; lane 3, MA1-D2; lane 4, MA1-B8; lane 5, MA1-B12; lane 6, MA2-A1; lane 7, MA2-E2; lane 8, MA4-C11; lane 9, MA6-F3; lane 10, MA7-A11; lane 11, MA7-B12; lane 12, MA8-G2; lane 13, MA8-F6; lane 14, MA10-D4; lane 15, MA10-E5; lane 16, MA10-A12; lane 17, MA12-G4; lane 18, MA12-G12. Molecular mass markers of 205, 116.5, 106, 80, 49.5, and 32.5 kDa (from top to bottom, respectively) were loaded on the left.

in acetone for 20 min at room temperature. Monoclonal antibody MA6-F3, diluted 1:500 in PBS, was incorporated into a micro-IF assay described above to detect *R. massiliae* in the smears. L929 cells infected with *R. massiliae* Mtu1^T were used as a positive control, and monoclonal antibody AF1-D12, specific for only *R. africae* and *R. parkeri* (60), was used as a negative control.

PCR-RFLP analysis. Template DNA was extracted from the remainder of the ticks by using the QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCRs incorporating two previously described primer pairs (*Rp* CS.877p-1258n and *Rr* 190.70p-602n) (22, 44) were used to amplify rickettsial DNA from these templates. In each amplification, DNAs extracted from *R. massiliae* Mtu1-infected L929 cells and noninfected L929 cells were used as positive and negative controls, respectively. The PCR amplification products were monitored by rapid electrophoresis (22, 47). DNA standard size marker 5 (Eurogenetec, Seraing, Belgium) was included in each run to estimate the molecular masses of the resolved products.

The PCR amplicons were subsequently identified by restriction fragment length polymorphism (RFLP) analysis (22, 44). Restriction endonuclease *Alu*I (10 U/μl; Boehringer Mannheim, Mannheim, Germany) was used for digestion of the *Rp* CS.877p-1258n amplification products (22), and *Pst*I (10 U/μl; Boehringer Mannheim) and *Rsa*I (10 U/μl; Boehringer Mannheim) were used for digestion of the *Rr* 190.70p-602n amplification products (44). The amplified product from *R. massiliae* Mtu1-infected L929 cells was included in these digestions. The restriction fragments were separated electrophoretically and were visualized under a UV transilluminator (22, 47). DNA standard size marker V (Boehringer Mannheim) was used to estimate the fragment size.

RESULTS

Production and characterization of monoclonal antibodies against *R. massiliae*. Fourteen days after fusion, a total of 163 hybridomas producing monoclonal antibodies against immunogen were obtained. Of these hybridomas, 16 were selected for limiting dilution. The corresponding monoclonal antibodies designated by the prefix MA were further characterized.

By the micro-IF assay, three monoclonal antibodies were species specific; three demonstrated extensive cross-reactivities with the large rickettsial panel: MA1-D2 reacted with all the rickettsial strains except *O. tsutsugamushi* and *C. burnetii*, MA1-B8 and MA7-B12 reacted with all species of the SFG, and the others showed reactivities with only a few SFG rickettsiae, including Bar29, "*R. aeschlimanni*," *R. rhipicephili*, *R. japonica*, and Israeli tick typhus rickettsia (Table 2).

Except for MA1-D2, the monoclonal antibodies could be characterized as follows: two belonged to the IgM class, two belonged to the IgG1 class, six belonged to the IgG2a class, three belonged to the IgG2b class, and two belonged to the IgG3 class (Table 2).

The specificity of this monoclonal antibody panel except for that of MA1-D2 was determined by Western immunoblotting (Fig. 1). Two monoclonal antibodies, MA1-B8 and MA7-B12, which demonstrated broad cross-reactivities with the SFG rickettsial species, were found to be directed against the lipopolysaccharide (LPS)-like antigen which exhibited a ladder-like pattern after the whole-cell lysate had been treated with proteinase K (Fig. 2). Monoclonal antibodies MA2-A1 and MA2-E2 are directed against epitopes located on the 106-kDa heat-labile protein. The others are directed against epitopes located on the 120-kDa heat-labile protein (Fig. 1 and 3).

The further analysis by isotyping indicated that all monoclonal antibodies which reacted with protein epitopes belonged to the IgG class, including the IgG1, IgG2a, IgG2b, and IgG3 subclasses, and that monoclonal antibodies directed against the LPS-like antigen belonged to the IgM class (Table 2).

Antigenic relationships revealed by monoclonal antibodies. All rickettsial species of the typhus group and the SFG rickettsia showed antigenic relationships, as demonstrated by monoclonal antibody MA1-D2, which exhibited extensive reactivities among rickettsial species. All species of the SFG exhibited clear antigenic relationships on LPS, as shown by anti-LPS monoclonal antibodies MA1-B8 and MA7-B12.

The 13 monoclonal antibodies directed against epitopes of surface proteins showed cross-reactivity with only a few SFG rickettsial species. Among these SFG rickettsial species, the GS strain (5) reacted with all 13 monoclonal antibodies; Bar29, "*R. aeschlimanni*," and *R. rhipicephali* reacted with six, five, and three monoclonal antibodies, respectively. Additionally, *R. japonica* and Israeli tick typhus rickettsia showed cross-reactivities with a few monoclonal antibodies (Table 2).

Antigenic relationships and the epitope distribution on proteins among the species of SFG rickettsia, which reacted with anti-*R. massiliae* monoclonal antibodies, were determined by Western immunoblotting (Fig. 4). Two major high-molecular-mass proteins, such as the 120- and 135-kDa proteins of Bar29, the 104- and 124-kDa proteins of "*R. aeschlimanni*," and the

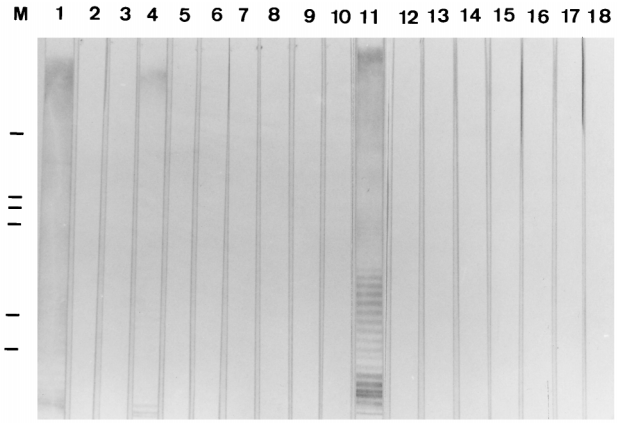

FIG. 2. Western immunoblotting of mouse polyclonal antisera and monoclonal antibodies with *R. massiliae* Mtu1^T proteins treated with 1.5 mg of proteinase K (Boehringer Mannheim) per ml at 37°C for 1.5 h. Lane 1, mouse polyclonal antisera; lane 2, noninfected mouse sera; lane 3, MA1-D2; lane 4, MA1-B8; lane 5, MA1-B12; lane 6, MA2-A1; lane 7, MA2-E2; lane 8, MA4-C11; lane 9, MA6-F3; lane 10, MA7-A11; lane 11, MA7-B12; lane 12, MA8-G2; lane 13, MA8-F6; lane 14, MA10-D4; lane 15, MA10-E5; lane 16, MA10-A12; lane 17, MA12-G4; lane 18, MA12-G12. Molecular mass markers of 205, 116.5, 106, 80, 49.5, and 32.5 kDa (from top to bottom, respectively) were loaded on the left.

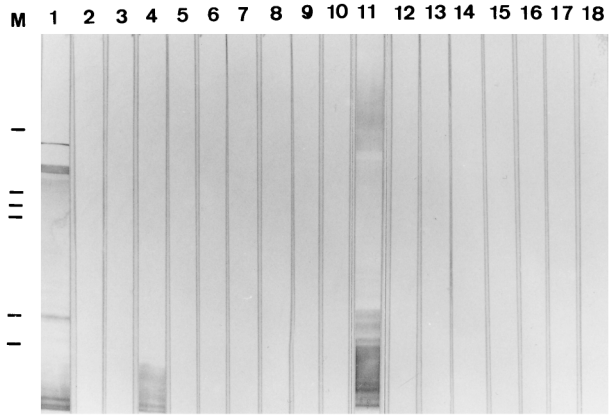

FIG. 3. Western immunoblotting of mouse polyclonal antisera and monoclonal antibodies with *R. massiliae* Mtu1^T proteins treated at 100°C for 5 min. Lane 1, mouse polyclonal antisera; lane 2, noninfected mouse sera; lane 3, MA1-D2; lane 4, MA1-B8; lane 5, MA1-B12; lane 6, MA2-A1; lane 7, MA2-E2; lane 8, MA4-C11; lane 9, MA6-F3; lane 10, MA7-A11; lane 11, MA7-B12; lane 12, MA8-G2; lane 13, MA8-F6; lane 14, MA10-D4; lane 15, MA10-E5; lane 16, MA10-A12; lane 17, MA12-G4; lane 18, MA12-G12. Molecular mass markers of 205, 116.5, 106, 80, 49.5, and 32.5 kDa (from top to bottom, respectively) were loaded on the left.

116- and 120-kDa proteins of *R. rhipicephali*, shared common epitopes with the 106- and 120-kDa proteins of *R. massiliae*. These two proteins were also detected with mouse polyclonal antisera (Fig. 1, lane 1). In addition, the 128-kDa protein of *R. japonica* (Fig. 4) also showed common epitopes with the 120-kDa protein of *R. massiliae* (Table 3).

Specific detection of *R. massiliae* in ticks. Among 20 *R. sanguineus* ticks tested, 3 of them were found to be infected with rickettsia-like organisms by using the species-specific monoclonal antibody MA6-F3 (data not shown). Detection with an anti-*R. africae* monoclonal antibody (AF1-D12) gave negative results for all ticks. Rickettsiae were also detected in these three ticks as well as another tick with a negative result by the micro-IF assay (faint band; data not shown) by PCR amplification. RFLP analysis of these amplicons yielded digestion patterns of products derived from the three micro-IF assay-positive ticks indistinguishable from those of *R. massiliae* (11, 22). The product from another tick detected only by PCR remained undigested after treatment with the restriction endonucleases used (data not shown).

DISCUSSION

The monoclonal antibody technique, which often reveals epitopes only weakly elicited in a polyclonal response, has proven to be a powerful tool in studying the antigenicity and virulence of rickettsiae (1–3, 25, 35, 57, 58). Monoclonal antibodies have now been produced against most of the pathogenic rickettsial species, including *R. prowazekii* (19), *O. tsutsugamushi* (40), *R. rickettsii* (1, 34), *R. conorii* (25, 35, 55), *R. sibirica* (35), *R. akari* (38), *R. japonica* (52), Israeli tick typhus rickettsia (58), and *R. africae* (60). However, the production of monoclonal antibodies against the rickettsial species of unknown pathogenicity toward humans has not yet been attempted. The antigenicity of these species has previously been analyzed only with mouse polyclonal antisera (11, 12, 43).

Previously, antigenic relationships among rickettsial species have been reported by using patient sera (41), mouse polyclonal antisera (16, 24, 43), and monoclonal antibodies (2, 3, 30, 57, 58). However, usually, only a few species like *R. prowazekii*, *R. rickettsii*, *R. conorii*, *R. sibirica*, *R. akari*, and *R. australis* were included in those studies. In our study, we included all the reference rickettsial species and *C. burnetii* in an analysis of their antigenic relationships using monoclonal antibodies raised against *R. massiliae*, an SFG rickettsia isolated only from ticks (13).

Most of the SFG rickettsiae (*R. africae*, *R. akari*, Astrakhan fever rickettsia, *R. australis*, *R. conorii*, *R. honei*, *R. helvetica*, *R. montana*, *R. parkeri*, *R. rickettsii*, *R. sibirica*, "*R. slovaca*," and Thai tick typhus rickettsia) shared only the common LPS antigen with *R. massiliae*, whereas the other species of the SFG rickettsiae (Bar29, "*R. aeschlimanni*," *R. rhipicephali*, *R. japonica*, and Israeli tick typhus rickettsia) shared epitopes on surface proteins as well as on LPS. These shared protein epitopes, which were located on both immunodominant proteins of *R. massiliae* (106- and 120-kDa proteins), were also demonstrated to exist on the surface proteins of Bar29, "*R. aeschlimanni*," *R. rhipicephali*, and *R. japonica* by Western immunoblotting. Among these species, Bar29 isolated from *R. sanguineus* ticks from the Catalan region (Spain) had the most epitopes in common with *R. massiliae*, concurring with previous studies on their phenotypic and genotypic characteristics (12, 47, 48).

Further analysis showed that many of the protein epitopes recognized were located on different-sized proteins in different species. The epitopes on the 106-kDa protein of *R. massiliae* were only found on the corresponding surface proteins of Bar29, "*R. aeschlimanni*," and *R. rhipicephali*, although epitopes on the 120-kDa protein were distributed more widely. These results suggested that some of the epitopes on the high-molecular-mass proteins of *R. massiliae* are shared among closely related rickettsial species. Furthermore, we found that some proteins (i.e., the 120-kDa protein of *R. massiliae*) which appear as predominant bands in protein profiles are also the principal immunogens in the humoral response of mice. However, some proteins (i.e., the 106-kDa protein of *R. massiliae*) which appear only as weak bands in protein profiles are probably strong immunogens in mouse immunization studies. A previous study of monoclonal antibodies against *R. africae* also demonstrated this phenomenon (60). Thus, the more promi-

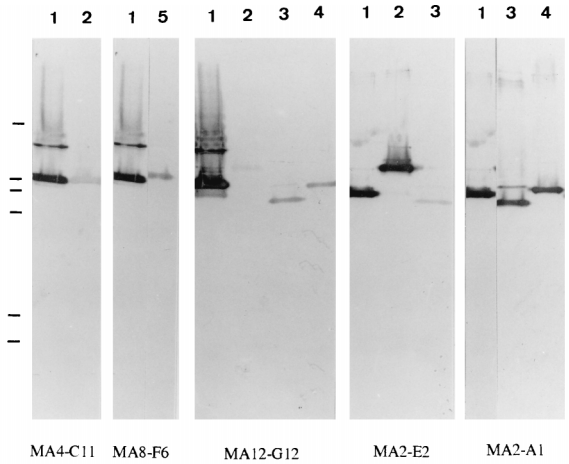

FIG. 4. Western immunoblotting of monoclonal antibodies with *R. massiliae* Mtu1^T and other SFG rickettsial strain. Lane 1, *R. massiliae* Mtu1; lane 2, Bar29; lane 3, "*R. aschalimanni*"; lane 4, *R. rhipicephali*; lane 5, *R. japonica*. Molecular mass markers of 205, 116.5, 106, 80, 49.5, and 32.5 kDa (from top to bottom, respectively) were loaded on the left.

TABLE 3. Antigenic relationships of surface protein of SFG rickettsiae exhibiting cross-reactivity with monoclonal antibody to *R. massiliae* Mtu1[Ta]

Monoclonal antibody	Isotype	Reactivity (kDa) of epitope on the protein of the following species[b]:				
		R. massiliae	Bar 29	"*R. aeschlimanni*"	*R. rhipicephali*	*R. japonica*
MA6-F3	IgG2a	120	–	–	–	–
MA8-G2	IgG3	120	–	–	–	–
MA4-C11	IgG2b	120	120	–	–	–
MA10-D4	IgG2a	120	120	124	–	–
MA2-E2	IgG1	106	135	104	–	–
MA2-A1	IgG2a	106	–	104	116	–
MA12-G12	IgG2b	120	135	104	120	–
MA7-A11	IgG2a	120	120	124	120	ND
MA8-F6	IgG2a	120	–	–	–	128

[a] The cross-reactivity of *R. japonica* and Israeli tick typhus rickettsia with monoclonal antibodies MA10-E5 and MA10-A12 cannot be detected.
[b] –, no reactivity; ND, not detected.

nent bands in the SDS-PAGE profile do not always correspond to the principal immunogens of rickettsial species.

In the present study, Bar29, "*R. aeschlimanni*," and *R. rhipicephali* also showed more antigenic similarities to *R. massiliae* than to other species of SFG rickettsia. These four SFG rickettsial species, which also exhibit close phylogenetic relationships (47, 48), could be considered one subgroup of the SFG rickettsiae.

R. massiliae has a unique epitope which stimulates the production of species-specific monoclonal antibodies. These species-specific monoclonal antibodies revealed the antigenic diversity between *R. massiliae* and other closely related rickettsial species. Furthermore, they also provide an alternative tool for identifying this species in ticks (60). In the past, identification of *R. massiliae* has relied either on its isolation (5, 11) or on PCR-RFLP techniques (6, 52). These two methodologies are both time- and material-consuming. For example, the identification of rickettsiae in 20 ticks by the PCR-RFLP technique requires 48 to 72 h and costs $3 to $4 per sample (51). By comparison, the detection of *R. massiliae* in the same number of ticks by a micro-IF assay incorporating a species-specific monoclonal antibody takes only 3 h and costs $0.30 per sample. The species-specific monoclonal antibodies raised from *R. massiliae* therefore appear to be a powerful and more acceptable tool for identifying *R. massiliae* in ticks in large-scale field and epidemiological work. Similarly, the use of a subgroup-specific monoclonal antibody can also be extended to identifying the species of the subgroup including *R. massiliae*, Bar29, "*R. aeschlimanni*," and *R. rhipicephali*.

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